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Oxidation reactions of phenolic extractive compounds in wood

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Thesis submitted for examination for the degree of Master of Science in Technology.

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Abstract

This thesis investigated the oxidation of phenolic extractive compounds in fresh wood immediately after the structure of wood is broken and the compounds come in contact with each other, ambient air and light at room temperature. Experimental part was conducted by sawing frozen birch wood to sawdust and then extracting it immediately with 80% acetone. Some sawdust was dried before acetone extraction and used as a comparison. Color of the sawdust samples was measured using CIELAB measurements and the reactivity of the samples was assessed by autohydrolysis trials. Extracts were analyzed using GC-MS, GC-FID, HPAEC and UV-vis spectrometry. There was a clear difference in the colors of the sawdust samples after acetone extraction. Dried samples had darkened visibly during drying and the extraction did not remove the colored compounds from the sawdust. Immediate extraction after sawing also yielded more extractives and the immediately extracted sawdust had less acetone soluble content after autohydrolysis than dried sawdust. Extract from immediately extracted sawdust contained (-)-epicatechin and sucrose that were present in extract from dried sawdust samples in significantly smaller quantities or in some cases completely absent. Conversely, glucose and fructose contents were higher in the extract from dried sawdust. A clear correlation between the disappearance of (-)-epicatechin in the extract and the discoloration during drying in sawdust could be seen. This enforces the theory that polyphenols are a key component in wood discoloration after felling.

Keywords wood extractives, phenolic compounds, discoloration, oxidation

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Tiivistelmä

Tässä opinnäytetyössä tutkittiin fenolisten yhdisteiden hapettumisreaktioita ilman ja valon vaikutuksesta puussa välittömästi puun kaatamisen ja sahaamisen jälkeen. Kokeellisessa osassa reaktioita tutkittiin sahaamalla jäisestä koivusta purua, joka uutettiin välittömästi asetonilla. Purua ja uuteaineita verrattiin samasta puusta tehtyihin verrokkinäytteisiin, jotka oli tehty uuttamalla puru asetonilla vasta sulatuksen ja kuivauksen jälkeen. Purujen väriä vertailtiin CIELAB-määrityksen avulla ja purujen reaktiivisuutta arvioitiin autohydrolyysikokeilla. Uutteiden koostumuksen määrityksessä käytettiin kromatografisia ja optisia menetelmiä. Purujen värimittauksissa havaittiin selkeä ero kuivattujen ja välittömästi uutettujen näytteiden välillä. Kuivatut näytteet olivat selvästi tummempia, eikä uutto muuttanut väriä merkittävästi. Kuivatun purun uutteessa oli myös vähemmän (-)-epikatekiinia ja sakkaroosia, mutta enemmän glukoosia ja fruktoosia, kuin välittömästi uutetun purun uutteessa. Purun tummumisen ja (-)-epikatekiinin vähenemisen välillä havaittiin selkeä yhteys, joka vahvistaa käsitystä fenolisten yhdisteiden tärkeydestä puun tummumisessa.

Avainsanat puun uuteaineet, fenoliset yhdisteet, värinmuutos, hapettuminen

PREFACE

This thesis was carried out from late 2018 to fall of 2019 at Aalto University. The funding was provided by FinnCERES Materials Cluster, of which I am thankful.

I would like to thank Kyösti Ruuttunen for providing me support in the experimental part and feedback on my writing. Kyösti has been the first person I go to whenever I don't know how to proceed, and he has always known what I should do next or been able to direct me to someone who does.

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For the support on autohydrolysis experiments I would like to thank Tainise Lourencon who taught me how to work with the reactor I used and who gave valuable insights into autohydrolysis during our discussions. Additionally, I would like to thank everyone working on FinnCERES FROG1 project alongside Tainise for their feedback and comments on my results. I hope your research is fruitful!

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Tomi Eilamo

Espoo, Finland, 26.9.2019

ABBREVIATIONS

BSTFA	N,O-Bis(trimethylsilyl)trifluoroacetamide
DDE	dark-dry-extracted
DE	dry-extracted
FE	fresh-extracted
GC-MS	gas chromatography with mass spectrometry
GC-FID	gas chromatography with flame ionization detector
HMF	hydroxymethylfurfural
HPAEC	high performance anion exchange chromatography
MC	moisture content
NDP	nucleoside diphosphate
NMR	nuclear magnetic resonance
OD	oven-dry
PA	proanthocyanidin
PPO	polyphenol oxidase
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TMCS	trimethylchlorosilane
UV-vis	ultraviolet and visible light

CONTENTS

1. INTRODUCTION

In addition to major structural polymeric compounds – cellulose, hemicelluloses and lignin – that make up most of wood, there is a myriad of different compounds in wood that can be extracted with organic solvents or water.[1] Many of these compounds are secondary metabolites that give resistance to herbivores and pathogens or function as frost hardening agents during winter.[1,2]

Phenolic extractive compounds in particular have been related to reactions in wood after felling. It is known that they go through autoxidative and enzymatic condensation and polymerization reactions in the presence of aerial oxygen and polyphenol oxidase (PPO).[3-5] Some phenolic extractives such as proanthocyanidins (PA) and their hydrolysis products, anthocyanidins, are also known to function as chromophores that enable coloring in plants and they are synthetized by plants for the purpose of exerting specific colors.[6-9] The connection between the colorimetric behavior of phenolic compounds in wood and their more spontaneous reactions after felling is however not entirely clear.

In addition to reacting with each other, phenolic extractives most likely react with other extractive compounds to form larger chromophores such as melanins that are significantly harder to extract than simple phenolic compounds.[3,4] This is a problem in further applications as extractives are considered detrimental in many processes and wood products, and utilization of lignocellulosic materials typically requires fractionation of the material to its main polymeric components.[1,10] In papermaking accumulation of phenolic extractives in process water may disturb the mill operations, in acid sulfite pulping extractives inhibit delignification[1] and in alkaline pulping they can cause scaling problems.[11] In mechanical wood products wood discoloration related to extractives causes loss in product value due to an undesired change in the shade of a product.[12]

In this thesis the extractive condensation and polymerization reactions occurring in wood were studied by extracting freshly sawn *Betula sp.* xylem sawdust with acetone to remove as much of the phenolic extractive compounds from the wood as possible before any reactions could occur. Freshly extracted sawdust was compared to first dried and then acetone extracted sawdust from the same tree. Sawdust samples were

optically analyzed for discoloration after extraction and the extracts were analyzed using various methods to establish a link between discoloration of wood and changes in the chemical composition of the extractives. Extractive-free sawdust samples were subjected to autohydrolysis and the products were gravimetrically analyzed to assess the effects of condensation and polymerization of extractives on the reactivity of wood.

Goal of this thesis was to show that oxidation reactions happen in wood after felling and to investigate the effects the reactions have on wood and wood extractives. The reactions were then connected to changes in chemical composition of extractives, the discoloration of wood and wood reactivity.

2. WOOD EXTRACTIVES

The non-lignocellulosic compounds that can be extracted from wood with various polar and nonpolar solvents are referred to as extractives.[1,13] This broad definition includes a wide variety of different compounds. It is therefore practical to categorize extractives further based on their structure. Harborne[14] categorizes extractable organic constituents in plants into six main categories:

- phenolic compounds
- terpenoids
- organic acids, lipids and related compounds
- nitrogen compounds
- sugars and their derivatives
- macromolecules

More specifically in wood, Sjöström[1] discusses extractives in four groups:

- phenolic constituents
- terpenoids and steroids
- fats and waxes
- inorganic components

Apart from fats, all the extractive groups mentioned by Sjöström[1] are a part of the secondary metabolism of plants. This means that they are not related to plant growth but have various other roles instead. This is also reflected on their distribution within a living tree, meaning that different parts of the tree have different extractives composition that is dictated by the desired function in each part.

Sugars, many of the nitrogen compounds such as amino acids, and many of the macromolecules such as proteins, are a part of the primary metabolism of a plant serving as source of energy or as a reserve food storage.[1,15] Extractable sugars, also called non-structural carbohydrates include mostly monosaccharides, disaccharides and starch. They mainly serve as energy storage for the tree and enable growth and respiration even when photosynthesis is not possible. They are also related to frost hardening during late fall and early winter.[2]

2.1 Phenolic compounds

Based on earlier research phenolic compounds are the most interesting group of extractives from the perspective of oxidation reactions and related discoloration in plants.[4,16-18] Phenolic compounds present in wood are known to oxidize both enzymatically and non-enzymatically in the presence of oxygen and light.[1,4] Phenolic compounds are further divided into five categories by Sjöström[1]:

- stilbenes
- lignans
- hydrolysable tannins
- flavonoids, including isoflavones
- condensed tannins (synonymous to PA)

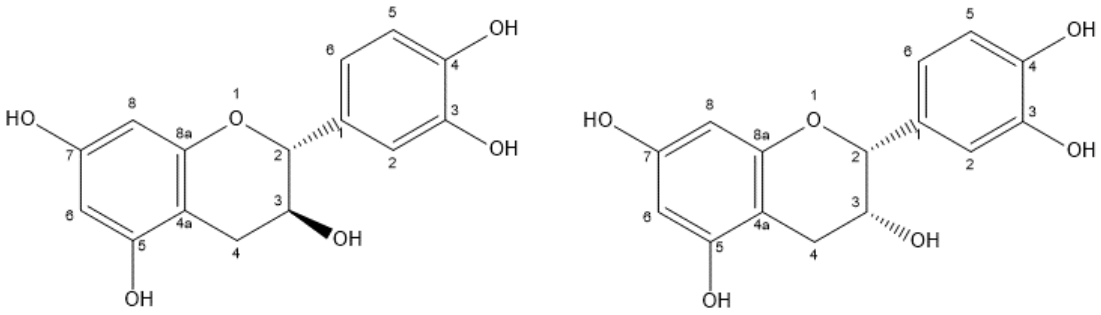
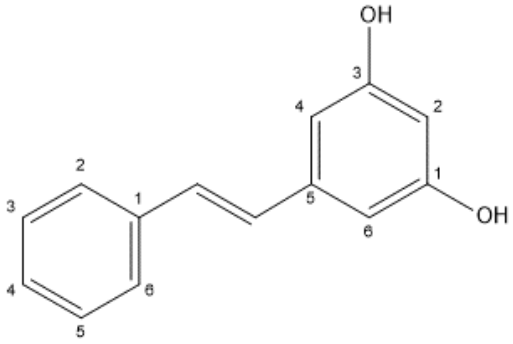
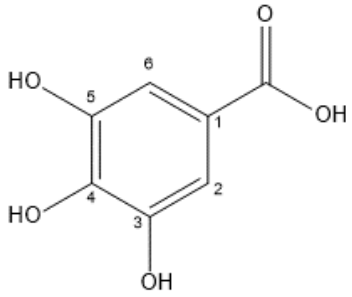


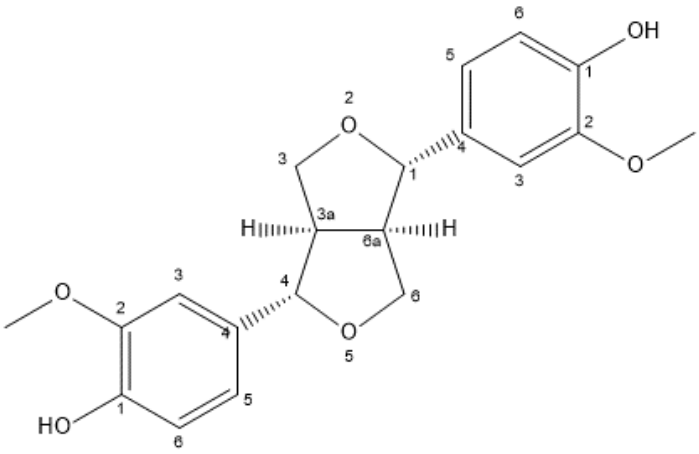
Figure 1. Two common flavonoids in wood, (+)-catechin and (-)-epicatechin. Proanthocyanidins (PAs) are oligomers and polymers of mainly these catechins and to smaller extent other flavonoids.[9]



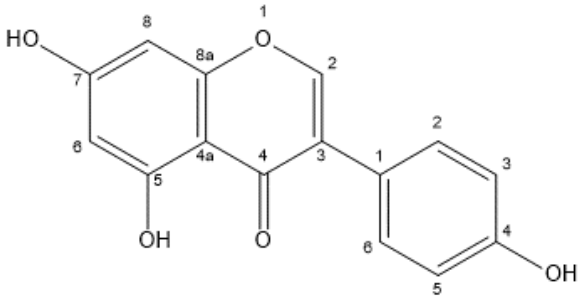
pinosylvin



gallic acid



pinoresinol



genistein

Figure 2. Examples of different phenolic compounds. Pinosylvin is a stilbenoid toxin synthetized by coniferous trees for defensive purposes.[19] Gallic acid is one of the two main constituents in hydrolyzable tannins.[1] Pinoresinol is a lignan found in some *Forsythia spp.* plants[20,21] as well as in *Betula pendula* Roth xylem.[22] Genistein is an isoflavonoid that can be found in many foods and medicinal plants.[23,24]

Stilbenes are 1,2-diphenylethylene derivatives with a conjugated double bond system. Lignans are formed by oxidative coupling of two phenylpropane units. Related to norlignans that have similar structure with one less carbon atom. Both stilbenes and lignans have fungicidal properties and they protect the tree from microbial attacks. They also contribute to the darker color of heartwood.[1,11]

Hydrolyzable tannins are typically esters of polyphenol carboxylic acids and D-glucose. They yield gallic and ellagic acids and sugars when hydrolyzed. Hydrolyzable tannins are not very common in trees.[1,11]

Flavonoids have a tricyclic diphenylpropane skeleton. Flavonoids, especially catechins, form PA, most typically 3-8 flavonoid units per oligomer. Isoflavones are structurally related to flavonoids but have slightly different coordination on their carbon skeleton from flavonoids as can be seen comparing genistein in Figure 2 to catechins in Figure 1.[1,11]

Phenolic extractives are toxic to many organisms, which enables plants to use extractives as a defense against herbivores and pathogens. Because of their toxicity, phenolic extracts are mostly coupled to sugars in plants to avoid harm to the plant itself. It is also speculated that phenolic compounds may function as growth inhibitors or as a nutrient storage during winter dormancy.[25] Phenolic extractives also affect the pigmentation of plants and protect plants from UV-B radiation.[18] On an ecosystem level it has been found that some polyphenols also have a strong influence on the degradation of soil organic matter and on nutrient cycling.[26,27]

Hiltunen et al.[22,28] have studied phenolic extractives in silver birch by extraction with methanol using Soxhlet apparatus and analysis with ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Based on their findings, birch contains at least 23 different compounds that classify as phenolic extractives. Phenolic extractive compounds were identified in birch trees from two different growing sites, one rich in nutrients and the other one scarce in nutrients. While the tree from the former site contained more total methanol-soluble extractives, extractives from the tree from the latter had a higher proportion of phenolic compounds. Most phenolic compounds were present in both trees in similar amounts with the exception of (+)-catechin 7-*O*-β-D-xylopyranoside which was present in the tree from the site scarce in nutrients in tenfold compared to the tree from the site rich in nutrients. Full list of phenolic compounds and their content in wood is presented in Table 1.

Table 1. Phenolic compounds extractable by methanol from birches in two different growing sites identified using 2D NMR spectroscopy. Catechins were found as (+)-catechin 7-O-β-D-xylopyranoside that was far more abundant in trees when nutrients were scarce.[22,28]

Compound	Content (mg g^{-1} dry wood)	
	Rich in nutrients	Scarce in nutrients
3,4,5-Trimethoxyphenyl-6-*O*-syringoyl-β-D-glucopyranoside	0.07	0.07
3,4,5-Trimethoxyphenyl-6-*O*-vanilloyl-β-D-glucopyranoside	0.11	0.05
4-Hydroxy-2-methoxyphenyl-6-*O*-syringoyl-β-D-glucopyranoside	0.09	0.13
2-Hydroxy-4-methoxyphenyl-6-*O*-syringoyl-β-D-glucopyranoside	0.13	0.08
4-Hydroxymethyl-2-methoxyphenyl-6-*O*-syringoyl-β-D-glucopyranoside and (+)-catechin 7-*O*-β-D-xylopyranoside	0.05	0.52*
Lyoniside	0.05	0.10
Nudiposide	0.05	0.07
(+)-Lyoniresinol 3-*O*-β-D-glucopyranoside	0.11	0.16
(-)-Lyoniresinol	0.02	<0.01
(-)-Syringaresinol	0.05	0.08
Aceroside VIII	0.05	0.16
(3*S*,5*R*)-5-(β-D-Glucopyranosyloxy)-1,7-bis-(4-hydroxyphenyl)-heptan-3-ol	0.02	<0.03
Platyphylloside	0.04	0.07
1,7-Di-(4′-hydroxyphenyl)-4-hepten-3-one	0.03	-
3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-propan-1-one	0.01	0.01
Syringaldehyde	0.03	0.01
Vanillin	0.01	0.01
Sinapyl aldehyde	0.01	0.02
Coniferyl aldehyde	0.03	0.01
3,4,5-Trimethoxyphenyl β-D-glucopyranoside	0.06	0.11
3,4,5-Trimethoxyphenyl β-D-apiofuranosyl-(1″→6′)-β-D-glucopyranoside	0.18	0.15
(+)-Syringaresinol-4′-*O*-β-D-glucopyranoside	0.02	0.03

*Mostly (+)-catechin 7-O-β-D-xylopyranoside.

2.2 Variation within wood structure

Structure of wood on cell level is a product of natural necessities of a living tree. Wood consists of cells that have different roles, most importantly strengthening, conducting and storing. Cells are interconnected by openings, pits, which allow transport of water and water-soluble compounds inside the tree. Cell types for each role vary between softwoods and hardwoods. Mechanical strength is provided by latewood tracheids in softwoods and by libriform fibers and fiber tracheids in hardwoods. Conduction of water and nutrients inside the tree is provided by earlywood tracheids and ray tracheids in softwoods and vessels and vessel tracheids in hardwoods. Lastly, storage is provided by ray parenchyma and longitudinal parenchyma cells in both softwoods and hardwoods. In softwoods additional storage is provided by resin canals that are surrounded by epithelial cells, which function as secreting elements.[29,30] From the per-

spective of wood extractives, parenchyma cells and resin canals are the most interesting parts of xylem as most of the phenolic extractives in xylem are stored in parenchyma cells while lipophilic extracts are deposited in resin canals.[30]

In most wood species the xylem includes two kinds of wood: sapwood and heartwood. Various lignans, flavonoids and PA, are widely distributed in xylem of both hardwoods and softwoods. Sapwood consists of mainly dead cells and is responsible for water transport in the tree, but also gives tree mechanical strength. Overall, sapwood is scarce in extractives but does typically contain some low molecular mass phenolic compounds. Most species form heartwood, a darker wood rich in extractives with no living cells. Heartwood gives tree mechanical strength and the extractives it contains make it resistant to microbes. Heartwood typically contains high molecular mass phenolic components like tannins and in *Pinus* species also stilbenes.[11] In xylem, most phenolic extractive compounds are mainly found in heartwood, but flavonoids are distributed throughout xylem.[1,11] In birch xylem extractive content is generally low, possibly due to no proper heartwood formation, but the content would seem to rise slightly from towards the top of the tree compared to the base of the tree.[2,31]

Main non-structural carbohydrates in the xylem of birch are starch, sucrose, glucose and fructose. Their concentration typically rises towards cambium and is smaller near the center of the tree. Heartwood by definition does not contain reserve materials such as starch.[2,32]

While xylem is the main focus of this thesis, it is important to note that bark typically has a higher phenolic extractive content than xylem and could therefore be a more feasible feedstock if phenolic extractive compounds were converted into products.[1,33] It is also notable that some phenolics are only produced as a response to tissue injury at least in the inner bark making the appearance of phenolics in different parts of wood even more complex.[34]

2.3 Seasonal variation

Liimatainen et al.[34] have studied the seasonal variation of phenolic extractives in inner bark of *Betula pendula*. Their results showed that there is no qualitative variation in phenolic composition that could be attributed to seasonal differences. It was also found that there are more significant quantitative variations between tree clones than

between seasons. Levels of many phenolic components were higher during winter than autumn, but the overall conclusion was that seasonal variation was small.

There is a significant seasonal variation in soluble sugars and starch in silver birch. The amounts of sucrose and starch are declining throughout spring and early summer and reach their lowest point at the end of summer. During autumn their amount rises all the way to early winter. Monosaccharides are at their highest after bud break in late spring and smallest mid-summer. The seasonal variation seems to be more pronounced in hardwoods than softwoods.[2,35]

3. DISCOLORATION REACTIONS OF WOOD

Change of color in wood and in wood-based products is a widely known phenomenon and several factors affecting the color change have been studied. Color change has been mainly linked to extractives and lignin in wood.[1,36,37] Extractive compounds in wood go through autoxidative and enzymatic reactions after felling and during wood storage. Double bonds in extractives are prone to attacks by oxygen and oxidation leads to chain reaction that produces reactive free radicals. Autoxidation reactions are also accelerated by light, particularly at UV wavelengths. Reactions are especially fast when wood is stored in smaller particle sizes. In some cases, wet storage conditions also enable faster proceeding conversion than dry conditions.[1,12]

Color change can often be seen with naked eye and documented with a camera, but for small changes in color and for acquisition of numerical data for the color of wood CIELAB scale is typically used. CIELAB is a triaxial color space, where the axes are named L, a, and b. “L” denotes the lightness of color from 0 (black) to 100 (white), “a” is green to red axis and “b” is blue to yellow axis. Typical scales for a and b are -100 (green/blue) to 100 (red/yellow).[38-40] CIELAB color space is presented in Figure 3.

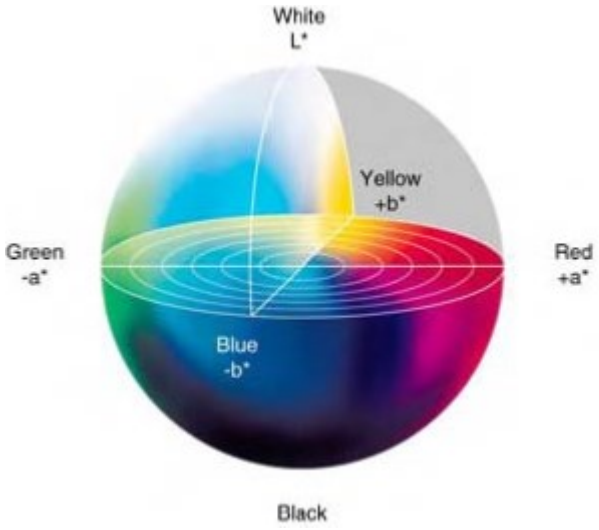


Figure 3. Three-dimensional graphical representation of the CIELAB color space.[41]

3.1 Enzymatic oxidation of polyphenols to melanins

Main enzymatic reaction of *o*-diphenols to *o*-quinones and the following non-enzymatic reaction to melanins is presented in Figure 4. The initial enzymatic reaction is reversible, but the following non-enzymatic reactions are irreversible. The main enzyme catalyzing the first reaction has been identified as polyphenol oxidase (PPO), but laccase is also known to catalyze the reaction. The main difference between the two is that PPO is selective to *o*-diphenols whereas laccase oxidizes other diphenols as well.[3,42] The first reaction can also occur without enzymes via autoxidation in the presence of oxygen and light.[1,12]

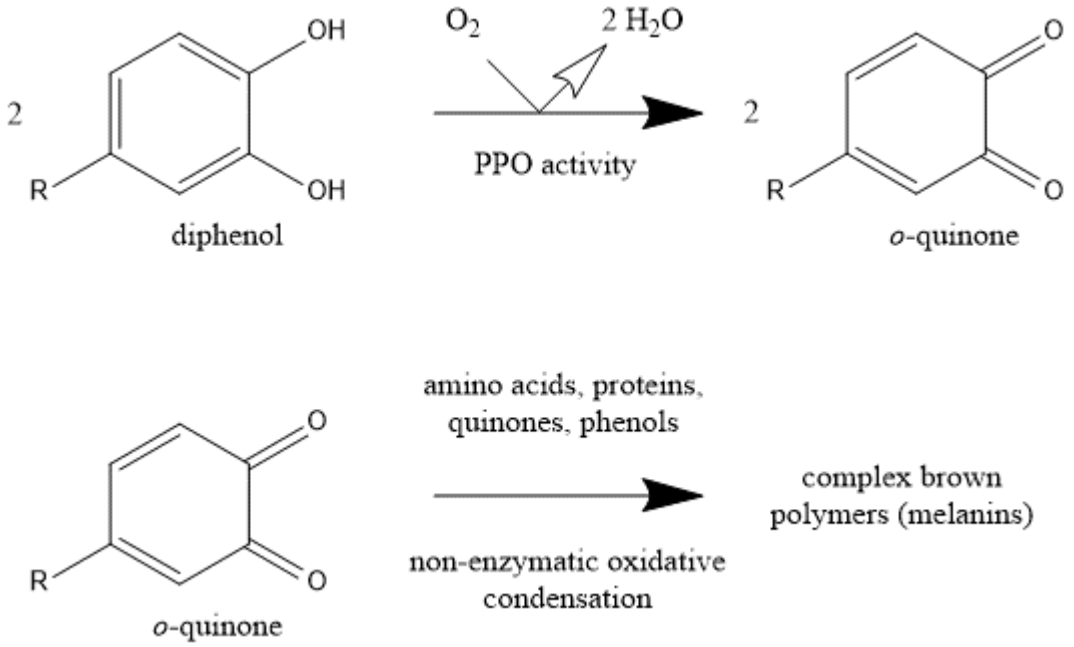


Figure 4. Simplified scheme of the enzymatic reaction of diphenols to quinones and further non-enzymatic reaction path to melanins that cause discoloration.[3]

Reversibility of the first part of the reaction is applied in some pulp bleaching processes. In pulping processes chromophores and potential chromophores are derived from phenols, catechols and quinones in combination with other extractive compounds. In reducing bleaching processes, reduction of *o*-quinones to their respective hydroquinones is used to increase the brightness of the pulp. Reduction can be achieved using sodium dithionite or sodium borohydride, but not with sulfurous acid due to its insufficient redox potential. Increased brightness achieved with this kind of bleaching is of course non-permanent as the hydroquinones are slowly converted

back to quinones through autoxidation in the presence of oxygen.[1] It was also found by Rouet-Mayer et al.[42] that in addition to reducing *o*-quinones back to *o*-diphenols, even the first polymerization products of the secondary reactions could be reduced by addition of ascorbic acid.

In the second part of the reaction various extractive compounds partake in *o*-quinone driven non-enzymatic condensation reactions resulting in variety of polymer compounds including melanins. Melanins are complex polymers that exert brown color in organic matter.[3,4] They have low solubility in water and most organic solvents making them difficult to extract from wood once they have formed.[43]

3.2 Enzymatic oxidation of polyphenols to proanthocyanidins

PA are synthetized by plants from catechins and other flavonoids. Very little is known of their biosynthesis pathways *in vivo*.[9] PA are known to function as chromophores in plants.[9,16,17] Hathaway and Seakins[5] conducted an experiment where PPO and (-)-epicatechin were combined and found compounds similar to PA being formed. Similar unregulated formation of PA could occur in wood when cell walls are broken after and during felling and flavonoids come in contact with enzymes and aerial oxygen. PA formation reactions in wood after felling are most likely similar to melanin formation reactions presented in Figure 4, but they only involve flavonoids and their *o*-quinones whereas melanins have more heterogenous substrates including amino acids, proteins and sugars.

Hiltunen et al.[16] studied the discoloration and the concentrations of phenolic compounds in different parts of a birch wood board before and after vacuum drying. They found that the amount of both PA and catechins was higher and the wood was darker on the surface of the board while the inner parts retained the natural lighter color of birch wood. The amount of (+)-catechin and (-)-epicatechin was also slightly higher on the surface than in the inner part. It was also stated that the discoloration is often amplified if the drying has complications and the moisture content of the wood stays high for an extended time.

Mitra[44] investigated the formation of conjugated polyphenols in tea leaves and found that prolonged oxidation leads to formation of yellow-brown PA or even compounds similar to lignin. The polymerization reactions he suggested were similar to those suggested by Yoruk and Ruhiye[3] for melanin formation that are shown in Figure 4.

3.3 Maillard reactions

In food sciences there are two common types of non-enzymatic discoloration reactions: caramelization and Maillard reactions. Both tie sugars to discoloration of organic matter. Caramelization is the dehydration of sugars that leads into polymerization that occurs at high temperatures. Maillard reactions occur typically at around 160 °C and involve mainly condensation of sugars with proteins and amino acids.[45] Maillard reactions are known to occur in birch sap during heating and they involve at least sugars and amino acids.[46]

Discoloration at elevated temperature is known to occur in wood. It is mainly accounted to products of condensation and oxidation of phenolic and polyphenolic compounds.[16,47] Wei et al.[47] found that there is a clear shift in color of locust wood towards darker, more red and more yellow, when treated at 120 or 140 °C. The shift in color was more pronounced at higher temperature, but the atmosphere in which the heat treatment was carried out had also a significant effect on the color development. In saturated steam atmosphere the discoloration was more pronounced than in oxygen and nitrogen atmospheres at same temperature. It was suggested that presence of water and oxygen could promote the oxidation and condensation reactions.

4. HYDROTHERMAL PRETREATMENT OF WOOD

Hydrothermal pretreatment of wood refers to various methods for hydrolysis of wood polymer components to improve wood fractionation. Hydrolysis conditions can be achieved with addition of acid or base, or as autohydrolysis in initially neutral conditions where only wood and water are treated with high temperature and pressure. While the autohydrolysis medium is initially neutral, it shifts to slightly acidic during the process due to formation of acetic acid from hemicellulose acetyl groups. Compared to acid-catalyzed hydrolysis, autohydrolysis requires higher treatment intensities.[48] The main benefits of autohydrolysis compared to acid or base treatments are lower environmental impact, good conversion of hemicelluloses to hemicellulosic sugars with various practical applications, less corrosion related problems and improved fractionation of cellulose and lignin. Autohydrolysis is also considered economically advantageous compared to alternative pretreatment technologies. Autohydrolysis, however, requires higher temperature and pressure than acid or base catalyzed hydrolysis pretreatments to compensate for the neutral pH, furthermore the formation of reactive lignin species that hinder the following fractionation processes has made autohydrolysis difficult to include in industrial scale processes.[10,49]

Hydrothermal treatment causes a variety of effects in wood, including extractive removal, hemicellulose hydrolysis and changes in the properties of cellulose and lignin.[10] Goal of the hydrothermal pretreatment is generally improving the fractionation of wood and producing lignin-, hemicellulose- and cellulose-based products from wood for further processing. For example, in the case of birch wood, the hemicellulose fraction is rich in polymeric xylan which is a promising material for barrier films, porous foams, hydrogels and coatings. In oligo- and monosaccharide forms xylose can be used as a raw material for chemicals, e.g. fuels and xylitol. Cellulose fraction can be used for viscose, acetate or paper grade pulp.[48] Hydrothermal pretreatment can also be used as a straight-up delignification process for birch wood. Borrega et al.[50] reached up to 80% delignification of birch wood by autohydrolysis at 240 °C.

Main reactions during hydrothermal pretreatment are hydrolysis and degradation of polymeric compounds in wood. Lignin goes through simultaneous degradation and condensation reactions. While the degradation reactions are faster than the condensation reactions, increasing the treatment temperature and time results in higher proportions of acid-insoluble Klason lignin to acid-soluble lignin.[50] Monosaccharides are

also converted into furfural and hydroxymethylfurfural (HMF) in acidic conditions. HMF can form a precipitate with lignin fragments and add to acid-insoluble Klason lignin fraction.[4,50,51]

The severity of autohydrolysis is often evaluated using either specified target temperature and time at that temperature or a variable called P-factor. P-factor is calculated from reaction time and temperature using Equation 1:

$$P = \int_{t_0}^{t} Exp\left(\frac{E_{A,H}}{R \cdot 373.15} - \frac{E_{A,H}}{R \cdot T}\right) dt, \qquad (1)$$

where $E_{A,H}$ is the activation energy 125.6 kJ · mol^{-1}, T is the temperature in K and R is the gas constant 8.314 J · K^{-1} · mol^{-1}.[52,53] P-factor evaluation excels over target time and temperature evaluation especially in cases where the rate of change in temperature during the heating and cooling of the reactor differs between batches as P-factor is cumulatively calculated throughout the process.

5. EXPERIMENTAL

Experimental part of this thesis is presented in Figure 5 and can be divided into three main parts. Firstly, producing sawdust and extracting it in different ways to show a difference in wood material based on exposure to oxygen, light and enzymes. These differences were verified with colorimetric analysis in CIELAB color space.

Secondly, verification of differences in extractive content with analysis of the chemical composition of the extracts. Gas chromatography with mass spectrometry (GC-MS) was used for qualitative analysis of phenolic compounds, gas chromatography with flame ionization detector (GC-FID) was used for quantitative analysis of phenolic compounds, high performance anionic exchange chromatography (HPAEC) was used for quantitative and qualitative analysis of sugars and ultraviolet and visible light (UV-vis) spectroscopy was used to determine the total aromatic soluble content of the extracts.

Finally, a study on effects of oxidation on autohydrolysis of sawdust to show if the differences in extractive content affect the reactivity of the wood. Reactivity in this case refers to selectivity and fractionation parameters of the autohydrolysis that were evaluated by formulating a mass balance for the autohydrolysis as well as a total mass balance that included the initial acetone extraction.

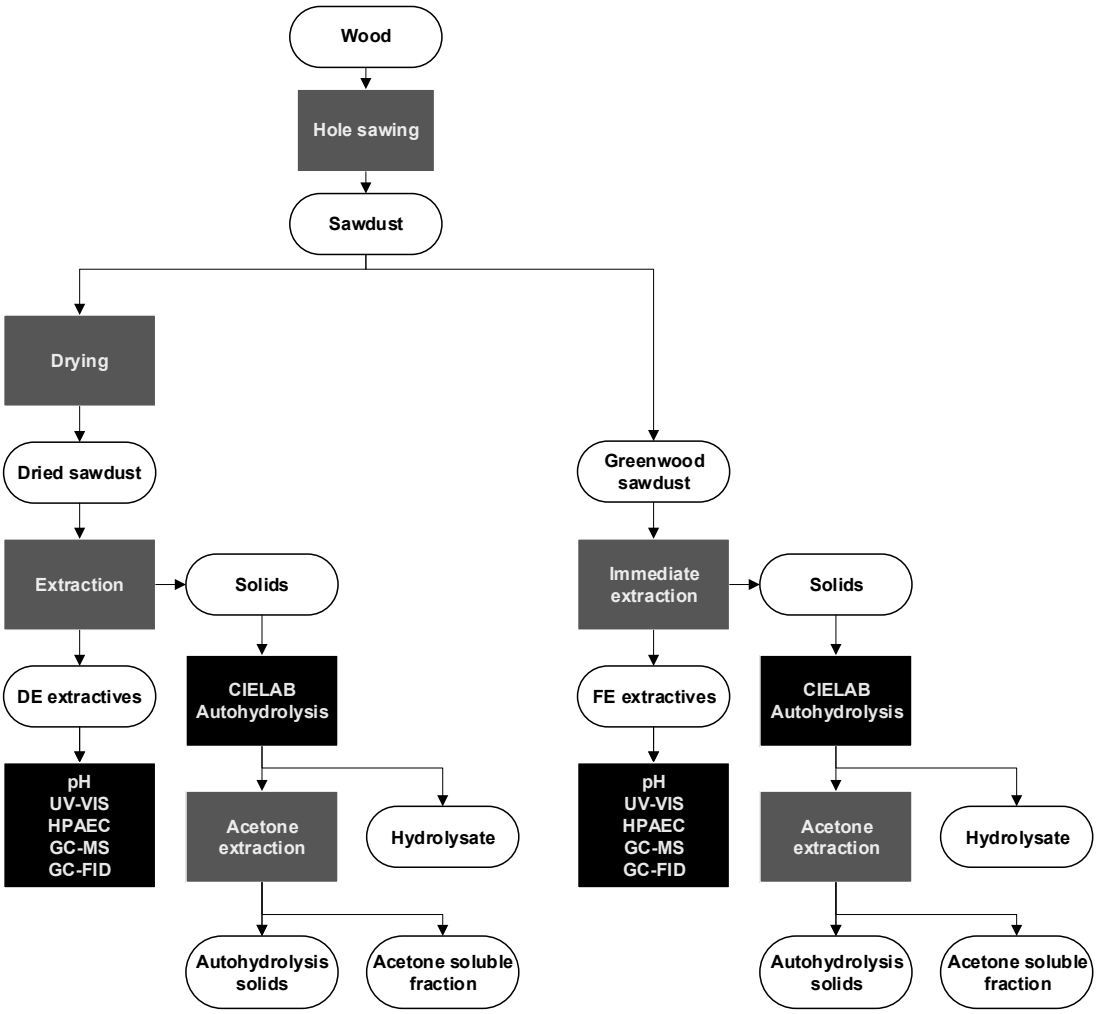


Figure 5. Flowchart of the experimental part of this thesis

5.1 Wood handling

Wood material used was from a single 18-year-old *Betula sp.* tree. Age of the tree was defined by counting growth rings. The tree was felled in Western Finland in early December during winter dormancy and sawn to 1 m long sections of tree trunk. Logs were wrapped in plastic immediately after felling to minimize interaction with aerial oxygen. Wood material was stored in a plastic bag at temperature below -18 °C before sawing. The material was then cut to 3-4 cm thick discs and sawn with Strands S53L pillar drill with a hole saw attachment that had diameter of 3.2 cm (Figure 6) at 105 rpm from both sides to produce sawdust. Both sapwood and heartwood were sawn while bark and knots were avoided.

Figure 6. The 3.2 cm diameter drill bit that was used to produce sawdust with a pillar drill.

Total of five batches were produced: two dry-extracted batches (DE1, DE2), two fresh-extracted batches (FE1, FE2) and a sample dried in dark before extraction (DDE). Batches and their main differences are presented in Table 2.

Table 2. Samples, their abbreviations and their main features.

Full name:	Fresh-extracted 1	Fresh-extracted 2	Dry-extracted 1	Dry-extracted 2	Dark-dry-extracted
Abbreviation:	FE1	FE2	DE1	DE2	DDE
Dried before extraction:			x	x	x
Covered from light:	x	x			x

In the case of DE1, sawdust was first collected to an empty 500 ml glass bottle. Some of the reference material was used for determining greenwood moisture content (MC) immediately after sawing. All MC in this thesis were determined according to SCAN-C 3:78, with minor adjustments to containers used during drying and measurements in some cases. The rest of the sawdust was stored in a loosely closed glass bottle for approximately 48 hours before it was spread out on a paper sheet and allowed to dry and interact with ambient air in a fume hood for a week. Dried sawdust was then transferred into a plastic bag and stored in room temperature. MC for dried sawdust was determined before extraction.

DE2 was transferred to fume hood for drying in ambient air immediately after sawing, giving it less time to stay moist after sawing compared to DE1. After a week of drying, sawdust was transferred to a plastic bag. DDE sawdust was dried for a week between

two large aluminum cups in dark lower compartment of a fume hood. It was transferred to plastic bag after drying.

FE1 and FE2 sawdust samples were immediately transferred from the wood disc to a 500 ml glass bottle covered with aluminum foil that contained acetone/water 4:1 ml/ml mixture that was used as an extraction medium. Acetone was provided by VRW and for water standard DI-water was used. Frozen wood discs were exposed to air for 25 minutes each during the sawing. Sawdust was exposed to air for maximum of 40 seconds between sawing and transfer from disc to the extraction medium. The amount of acetone and water were calculated before sawing to match the ratios used for reference material based on greenwood moisture content that was determined earlier, and the bottle was weighed between sawdust additions to obtain similar amount of oven-dry (OD) material as in DE batches.

5.2 Extraction and filtration

All sawdust samples were extracted in room temperature in a glass bottle with liquid/OD wood ratio of 20:1 ml/g and acetone/water ratio of 4:1 ml/ml. For DE and DDE sawdust samples the wood was transferred into a 500 ml glass bottle and water and acetone were then added in aforementioned ratios. For DDE sawdust the bottle was covered with aluminum foil to prevent light catalyzed reactions. Extraction was carried out for 25 hours from the acetone addition. As mentioned earlier, immediately after sawing the FE sawdust samples were transferred into an aluminum-foil-covered 500 ml glass bottle containing premeasured amounts of acetone and water. Because the sawdust was added to extraction medium over a 50-minute time period, the extraction was carried out for 23 hours and 35 minutes from the last sawdust addition to achieve an average extraction time of 24 hours for the whole batch. Sawdust suspensions were magnetically stirred with P-Selecta Agimatic-N stirrer at 700 RPM during the extraction to enable more homogeneous extraction.

After extraction, sawdust samples were immediately filtered in Büchner funnel with Whatman 1 filter paper. Cakes were washed with 50 ml of acetone twice. Washing liquid was included in the extract solution to maximize the yield of extractives. Cakes were dried in ambient air in fume hood spread on an aluminum plate over 5 days. Extracts were transferred into plastic bottles that were squeezed to remove as much air as possible and then closed tightly. Extracts were then stored in refrigerator at 6.5

°C and in the case of FE and DDE extracts covered with aluminum foil. DE1 and FE1 extracts were moved to freezer after 5 days of refrigeration to further retard and prevent any reactions. DE2 and FE2 extracts were concentrated and lyophilized immediately after extraction.

5.3 Concentration and lyophilization

Acetone was evaporated from dilute extracts with rotary evaporator (Büchi R-210, V-850, V-700) at 35 °C, 160 mbar. Temperature and pH at 25 °C were measured for the concentrated extracts after evaporation with Mettler Toledo Seven Compact.

After evaporation, concentrated extracts were moved to 100 ml plastic containers with known weight, frozen overnight and lyophilized with LABCONCO FreeZone 2.5. Containers were weighed after lyophilization to determine the yield of extractives from sawdust.

Due to some sawdust not being filtered in DE2 and FE2 extracts, those two extracts were resolubilized in acetone and water after lyophilization and centrifuged at 3000 rpm for 10 minutes to remove all sawdust. They were then concentrated and lyophilized again.

5.4 Autohydrolysis

The extractive-free FE and DE sawdust samples were autohydrolyzed in a 50 ml Parr 4841 reactor with stirring and heating. Liquid to solid ratio was 8:1. Target temperature was 205 °C and target P-factor was 800. P-factor was approximated during the autohydrolysis by calculating it every minute cumulatively using Equation 1. Six autohydrolysis runs in total were completed, one for each DE sample and two for each FE sample.

After autohydrolysis, the hydrolysate was separated from the solids with porosity 4 sinter glass. Hydrolysate was stored in freezer. Autohydrolyzed solids were washed with water until the washing liquid was clear. Acetone-soluble lignin was then extracted from the solids with 90-vol% acetone using the same sinter glass until the liquid was clear. DE1 and FE1 autohydrolyzed solids were dried at 105 °C before the lignin extraction, while DE2 and FE2 were immediately extracted after autohydrolysis and washing.

After extraction, lignin solution was transferred to a flask with known weight. Acetone was evaporated from the lignin solution using a rotary evaporator at 40 °C and the remaining acetone and water were evaporated in a vacuum oven at 40 °C. Amount of acetone-soluble lignin was determined gravimetrically. Dried lignin was stored at room temperature. Washed and extracted autohydrolysis solids were dried at 105 °C to determine their OD mass. Masses of solids and acetone-soluble lignin were subtracted from the OD mass of the sawdust used to determine the OD mass of hydrolysate. Along with the mass of lyophilized extracts, these masses were used to formulate the total mass balances for the autohydrolysis trials.

5.5 Analyses for sawdust

After the extraction and drying in fume hood, color of the sawdust for all samples was analyzed using L&W Elrepho. Cakes for color measurement were prepared from sawdust by preparing a suspension of 0.33 g (OD) of sawdust in 84 ml of water and filtering it with 45 mm diameter Büchner funnel with a Whatman 4 filter paper. Cake was then laid down on absorbent papers and another filter paper with absorbent papers were laid on top of the cake. Cakes were pressed in a sheet press twice at 2 bar pressure for 1 min and the top filter paper was detached from the cake after each pressing. After pressing, cakes were left to acclimatize in standardized paper measurement room overnight and measured the next day.

5.6 Analyses for extracts

Sugars in the extracts were quantified using Dionex ICS-3000 HPAEC. Sample preparation was mostly based on NREL/TP-510-42618, but acid hydrolysis was not carried out as it would have caused sucrose and other disaccharides to be cleaved to monosaccharides. 300±10 mg (OD) of extracts were weighed to plastic containers and 87 ml of water was added. Solutions were sonicated for 15 minutes and then magnetically stirred overnight at room temperature to dissolve as much of the extractives as possible. The next day 1:10 and 1:50 solutions were prepared from dissolved extracts with water. Approximately 1.5 ml of solutions were injected through a filter to HPAEC vials.

Gas chromatography to identify and quantify phenolic substances was conducted with Shimadzu GC-MS with Optic 4 and Shimadzu GC2010 FID. Samples were prepared

by weighing 4.5-5.5 mg of lyophilized extracts in a glass container and solubilizing them in 2 ml of pyridine per 1 mg of extract. 1.0 ml of solutions were transferred to GC-vials and 0.2 ml of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) 95:5 ratio solution was added as silylation agent. After the addition of silylation agent, solutions were sonicated for 10 minutes at room temperature. (-)-Epicatechin (Sigma Aldrich) was used as an external standard for peak identification. Sugars in the extracts were quantified with HPAEC and glucose was used as an internal standard for quantification of (-)-epicatechin from GC-FID peak data. An external standard with glucose was used to determine response factor for (-)-epicatechin and to aid in peak identification.

UV-vis spectroscopy to quantify soluble aromatic compounds was conducted using Shimadzu UV-1800 spectrophotometer. 100 mg/l solutions were prepared from extracts in 60 ml plastic containers with ethanol/water 1:1 ml/ml and sonication at room temperature. Spectra of extract solutions were measured in UV cuvette, and the spectrum of the cuvette and pure solvent was deducted from the total spectra. Amount of soluble aromatic compounds was calculated from the spectra using Beer-Lambert equation with the absorptivity of 20 $dm^3\ g^{-1}\ cm^{-1}$ at 280 nm.[4] Effect of baseline variation among samples was eliminated by deducting the absorbance for each sample at 300 nm from the absorbance at 280 nm. Equation 2 was used for calculating the concentration:

$$c = \frac{A_{280nm} - A_{300nm}}{\varepsilon \cdot l}, \qquad (2)$$

where c is the concentration of total soluble aromatic compounds in g/dm^3, A is the measured absorbance at corresponding wavelength minus the absorbance of pure solvent sample at that wavelength, ε is the absorptivity and l is the length that the light travels in the measured medium in cm.

6. RESULTS AND DISCUSSION

6.1 Wood handling

The amount of sawdust used for each batch was in the range of 12 to 15 g (OD). After the sawing some of the sawdust stuck to the surface of the wood disc. This would suggest that some of the water in the sawdust melts from the mechanical pressure applied by the sawblade and the moist sawdust then freezes again on the cold surface of the disc. Because of this, it is possible that some rapid reactions that require liquid water in wood may occur even in FE sawdust samples before extraction can take place. For future experiments the frozen wood could be either sawn while already submerged in the extraction medium or liquid nitrogen could be used to cool down the wood during sawing or milling. Both methods would also minimize the contact of wood with aerial oxygen even further.

Cracking of the wood by sawing produced some larger wood chips into the sawdust. Size of the larger chips was visually approximated as 10-20 times larger than most of the sawdust produced without cracking. It is, however, unlikely that the heterogeneity of the sawdust would be a significant factor in the later stages due to a long extraction time of 25 hours.

6.2 Extraction and filtration

Extraction mediums were successfully prepared apart from FE2, where slightly too much acetone and water were used resulting solid/liquid ratio of 22:1. Effect of ratio is however very small in this area and it should not have a significant effect on the extraction.

There was a clear difference in the colors of the DE and FE sawdust samples after extraction. As seen in Figure 7, after extraction and drying in ambient air the DE sawdust samples were darker while the FE sawdust samples had color similar to freshly cut birch wood. This color difference would suggest that a significant portion of the substances responsible of the color change typical to birch were extracted before the color change reactions could occur.

Figure 7. DE1, DDE, DE2 and FE1 sawdust samples after extraction. (Photo: Valeria Azovskaya, 2019)

There was no visible difference in the sawdust before and after extraction, but the extraction media were lightly colored with all samples. This would suggest that most chromophores were not extracted by acetone and remained in the sawdust after extraction. Discoloration is discussed in more detail in subchapter 6.5.

6.3 Concentration and lyophilization

After the concentration by rotary evaporation, pHs of the extract solutions were very similar for FE1 and FE2 at 5.24 and 5.22. For DE extract solutions there was a significant difference as the pH of DE1 was 4.19 while the pH of DE2 was 5.15. Because of its similarity in color with DE1, DDE batch was also measured for pH. Measured pH for DDE extract solution was 5.09, leaving DE1 as a clear outlier. It is unclear what could cause the difference in pH but considering the significant discoloration in DE1 sawdust the pH change may be related to the discoloration reactions.

Dissolving in acetone and water again for centrifuging to remove solids after the first lyophilization made the texture of second extract batches after the second lyophilization very different from the first batches that were not centrifuged and only lyophilized once. The first batches were sticky paste while the second batches were more powdery (Figure 8). It is unclear what the cause for this was, but it is most likely related to changes in the extracts during the first lyophilization and the following resolubilizing with 80% acetone rather than the centrifugation itself.

Figure 8. From left to right: dried extracts of DE1, FE1, DE2 and FE2. There is a clear difference in the texture between the first batches and the second batches.

6.4 Autohydrolysis

Due to limitations in the reactor heating and cooling controllability, peak temperatures, reaction times and P-factors among batches have some variance. Target temperature for the reactor was 205 °C in all batches, but it was not quite reached with some batches due to heating problems. Because of the variance in heating among batches, either the mixing was insufficient or that the heating system was not functioning as intended in all batches. Peak temperatures were in the range of 199 °C to 206 °C and the P-factors were in the range of 774 and 843. It is therefore reasonable to assume that some variance in the results may be due to variance in the reaction conditions. Some differences could, however, be seen between DE and FE mass balances. Averages of DE and FE mass balances are presented in Figure 9.

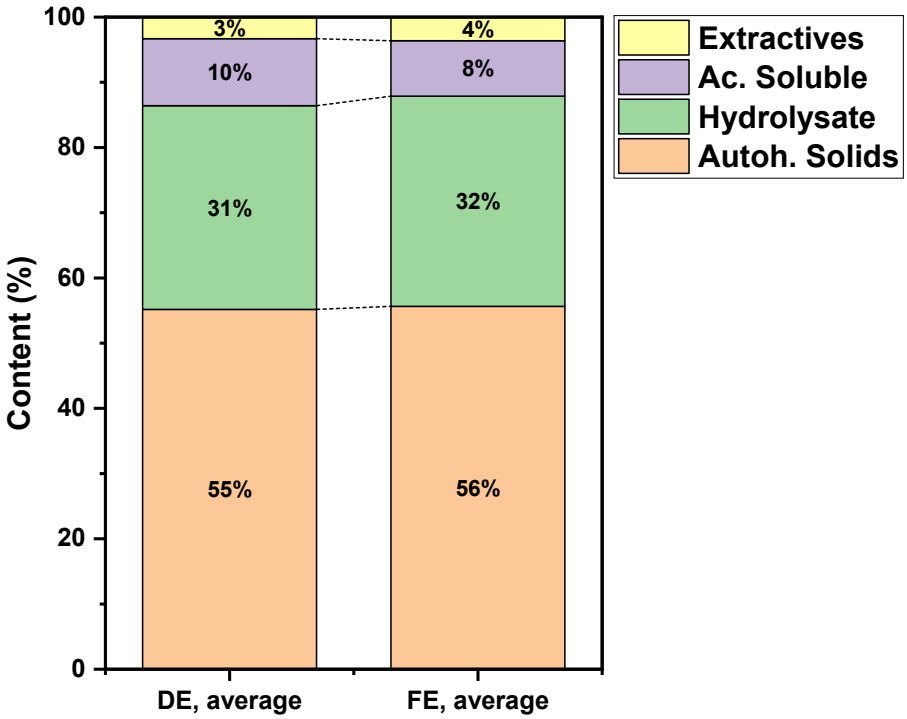


Figure 9. Averages of OD mass balances of DE and FE batches. Initial wood material was separated into four parts: extractives that were extracted with acetone before autohydrolysis, acetone soluble part after autohydrolysis, dried hydrolysate and autohydrolysis solids. Most notable differences are in the amount of extractives from the initial acetone extraction done before autohydrolysis and the amount of acetone soluble content after autohydrolysis.

The acetone soluble fraction in DE sawdust samples on average was 10.3% while in the FE sawdust samples the average was 8.5%, with 0.3% and 0.8% standard deviations, respectively. The amount of OD material in hydrolysate, on the other hand, was higher in FE sawdust samples except for the first FE1 batch that had the lowest hydrolysate fraction of all batches (27.0%), while the average for other FE sawdust samples was 34.0% and for the DE sawdust samples 31.2%. The proportion of solid fraction after autohydrolysis and subsequent acetone extraction was similar in DE and FE batches apart from the first FE1 batch that had 60.1% solids compared to the 52.9% to 55.9% range of the other batches. It should be noted that hydrolysate in mass balance calculations also includes losses in handling and transfer of the autohydrolysis products as it is calculated by subtraction of other fractions from the total initial mass.

Based on slightly lower amount of acetone-extractable content before autohydrolysis and higher amount after autohydrolysis, it seems that compounds that are initially extractable with acetone become insoluble during drying. In autohydrolysis conditions

their structure changes and they can be extracted with acetone after the autohydrolysis. Acetone soluble part after autohydrolysis contains typically high amounts of lignin. It is possible that the extractive compounds have formed bonds with lignin during drying and become acetone soluble similarly to lignin during autohydrolysis. However, this is not the only option, as melanin pigments are also insoluble in acetone[54] and can most likely therefore survive the initial acetone extraction in dried sawdust even without bonds to structural polymers of wood.

6.5 Analyses for sawdust

CIELAB color coordinates, as well as their respective color with 24-bit RGB resolution, are presented in Table 3. The conversion from CIELAB C 2° to RGB format was achieved using a free converter available at https://www.nixsensor.com/free-color-converter/. Based on the CIELAB data, the color of FE birch wood is significantly brighter, less red and less yellow than the color of all DE and DDE samples. DE1 and DDE samples possessed the darkest color with DDE being slightly more red and more yellow than DE1. DE2 sitting between DE1 and FE sawdust samples in all color coordinates would suggest faster drying prevents the discoloration reactions to some extent but not as effectively as fast immersion in the extraction medium.

Table 3. Numerical CIELAB C 2° color values for sawdust samples after extraction and visual presentation of corresponding colors in 24-bit RGB system resolution.

	DE1	DE2	FE1	FE2	DDE
L*	80.9	86.0	89.2	90.1	80.8
a*	1.1	0.0	-0.9	-0.8	1.8
b*	21.9	16.9	10.2	10.0	22.3

The colors of DDE and DE1 samples are likely similar because both samples dried more slowly than DE2 sample due to mass transfer limitations posed by the light cover in the case of DDE and storage in a bottle before drying in the case of DE1. During the drying of DE2, the surface color remained light, but under the surface the sawdust was darker, having a similar color with DE1 and DDE. In all cases where mass transfer with ambient air, and therefore evaporation of water, was hindered, the discoloration advanced further. Presence of water in the wood material allows aqueous reactions to take place. It would therefore seem that the discoloration reactions, whether they

are autoxidative or enzymatic, require an aqueous solution. It has also been previously reported that unsuccessful drying can amplify discoloration in birch board in a magnitude similar to what was seen between DE1 and FE samples.[16] This implies that sufficient rate of drying is extremely important in prevention of discoloration if phenolic extractives are not separated from wood material before drying.

Because the discoloration proceeded furthest in samples that had least contact with light and oxygen, it could be claimed that they are not important in the oxidation reactions. In the case of light this is most likely true as DDE sample was among the darkest with DE1. To determine the importance of light more extensively the effect of enzymes should be eliminated. In the experiments conducted in this thesis there was no control over the oxygen in samples. There are, however, several studies where the oxygen uptake has been monitored and found a relevant parameter in discoloration.[4,5,42] Based on this experiment, it can be concluded that the amount of oxygen required for discoloration reactions is very small and therefore prevention of discoloration reactions by removal of oxygen is most likely inconvenient in comparison to immediate immersion in acetone or other similar extraction medium.

The change in color is in line with colorimetric behavior of *Betula pendula* xylem sap obtained by mechanical compression in a recent study by Yamamoto et al.[4], who reported a significant change in the color of the sap over a 1000-minute period and a corresponding increase in absorbance in 300-600 nm wavelength range in UV-vis measurements. While producing sap by mechanical compression is in many ways different from producing sawdust, mainly because the sap is completely separated from the solid wood material in compression, the two methods share some important similarities. In both methods the structure of the birch wood breaks under mechanical stress allowing the various chemical compounds present in different morphological sites of wood to interact with each other, ambient air and light. The discoloration also takes place in room temperature in both cases. Because the discoloration is similar, it is reasonable to assume that these similarities in the methods, or at least some of them, are the key factors in this type of discoloration.

The discoloration of sawdust samples occurred in room temperature. This would point to reactions being mainly enzymatic, since purely non-enzymatic reactions, such as Maillard reactions, typically require significantly higher temperatures. In addition to removing (-)-epicatechin from the sawdust, the immersion in acetone also most likely

inactivated PPO and any other enzymes that are required for fast condensation reactions in room temperature. It has been previously found that replacing water with other solvent generally decreases the rate of polyphenol oxidation reactions, especially if the enzymes are directly suspended in mostly nonaqueous medium without first dissolving them in water.[44,55] Because of this, the FE sawdust samples remained exceptionally bright in color as no discoloration reactions occurred in extraction medium.

6.6 Analyses for extracts

HPAEC sugar analysis results show a clear correlation with the amount of sucrose in extracts and the color of the sawdust. As seen in **Virhe. Viitteen lähdettä ei löytynyt.** and Figure 10, the amount of sucrose is largest in FE extracts, slightly smaller in DE2 and below detection range in DE1. In addition to the lack of sucrose, DE1 is distinguished from other samples by the largest galactose content and being the only sample with enough xylose to reach the detection range.

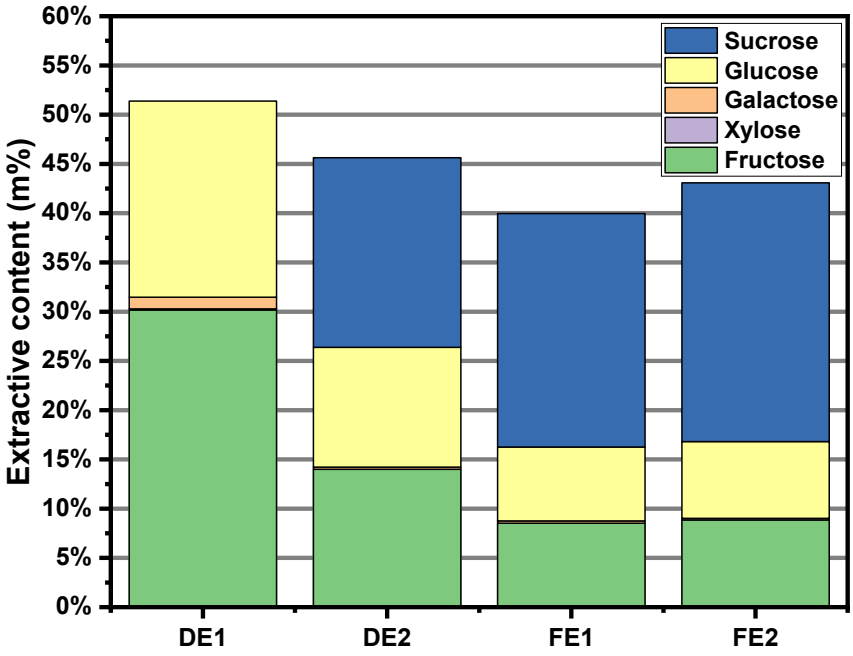


Figure 10. Relative contents of sugars in the extracts of each sample from HPAEC analysis. All monosaccharides where found in larger quantities in DE samples, especially in DE1, whereas sucrose was more abundant in FE samples and not found at all in DE1. Xylose was only found in DE1 at 0.1 m%.

Higher glucose and fructose contents in DE samples compared to FE samples can be explained by hydrolysis of sucrose. Cleavage of sucrose in plants is catalyzed by two enzymes: invertase and reversible sucrose synthase. Invertase catalyzed reaction produces glucose and fructose from sucrose, whereas sucrose synthase catalyzed reaction includes nucleoside diphosphates (NDP) as substrates in addition to sucrose and produces NDP-glucose and fructose (Figure 11).[56,57]The amount of glucose was disproportionally small compared to the amount of fructose in DE samples which would suggest that there was some sucrose synthase activity and some of the glucose in extracts is in NDP-glucose form.

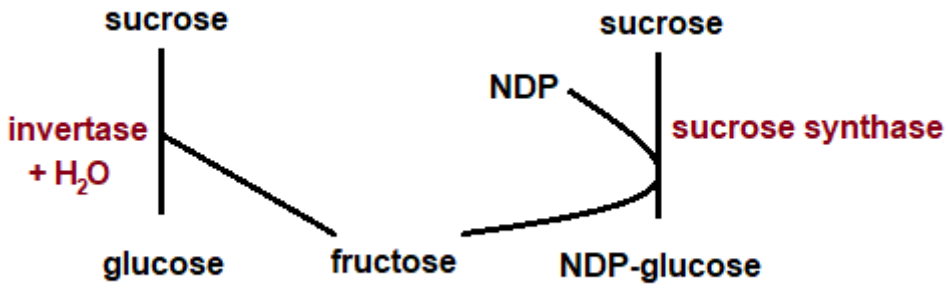


Figure 11. Two known alternative *in vivo* cleavage pathways of sucrose.[56,58]

The most significant differences in FE and DE batches were in the amount of sucrose and (-)-epicatechin. Both compounds were present in FE and in slightly smaller quantities in DE2, but absent in DE1. It is likely that the discoloration reactions consume (-)-epicatechin, but whether cleavage of sucrose is related to discoloration reactions is unclear. A correlation between high sucrose content and darkening in storage over time has been found in potatoes. Hypothesis proposed was that sucrose is available for conversion to reducing sugars that are then prone to non-enzymatic browning reactions with amino acids. These browning reactions, however, where only observed at elevated temperatures.[59]

The amount of (-)-epicatechin was the highest in FE2 at 3.0% of the total OD mass of extract and lowest in DE1 where it could not be identified at all using GC. This is quite different from findings of Hiltunen et al.[16] on birch boards during vacuum drying as they measured slightly higher amounts of (+)-catechin and (-)-epicatechin on the darker surface wood of the board than on the lighter inner parts of the board. In this experiment the drying was conducted without vacuum or elevated temperatures

Based on the GC-MS database compound recognition, small amounts of another flavonoid called taxifolin was found in both FE samples and DE2. Taxifolin is not a common phenolic extractive in birch, but taxifolin derivatives have been previously identified in birch wood.[60] Like (-)-epicatechin, taxifolin has *o*-diphenol structure that can be oxidized to a more reactive *o*-quinone and could therefore play a role in the discoloration.

Total content of the identified sugars increased from FE batches to DE2 and even more to DE1. This would suggest that there was little, if any, consumption of glucose and fructose during drying. The main substrates for the discoloration reactions are therefore phenolic compounds, mainly (-)-epicatechin, and possibly amino acids and proteins. Total amount of sugars was, however, high in all extracts making it difficult to completely rule them out as a substrate in discoloration reactions as they would not have been a limiting factor in any reactions unlike (-)-epicatechin that was consumed during drying.

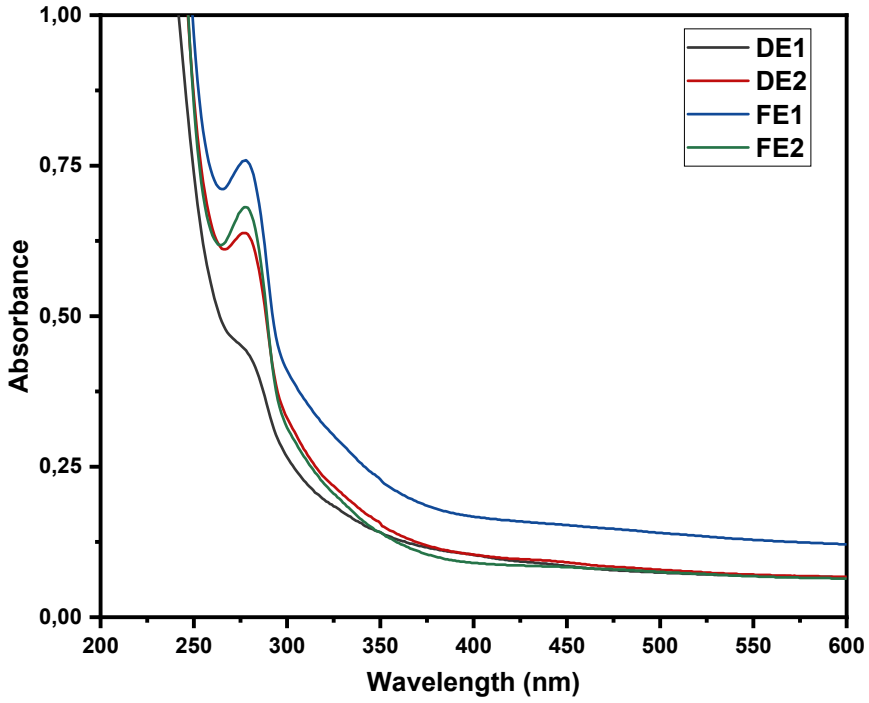


Figure 12. UV-vis spectra of extracts.

UV-vis spectra of extracts with the spectrum of solvent and cuvette deducted is presented in Figure 12. In all spectra there is a significant absorbance between 275 nm and 280 nm. The absorbance is largest in FE1 and slightly smaller in FE2. DE1 has the smallest absorbance maximum while DE2 sits between FE2 and DE1. Soluble aromatics including catechins are the main cause of the absorbance peak as they have an absorption maximum in this wavelength area. The concentrations of the total soluble aromatics in the extract solutions for DE1, DE2, FE1 and FE2 are respectively 6.0 mg/l, 12.6 mg/l, 14.7 mg/l and 15.6 mg/l. The amounts of total soluble aromatics from UV-vis spectroscopy and (-)-epicatechin determined from GC-FID data are included in Table 4.

Table 4. Summary of the composition of extracts. Amounts in mg g^{-1} of OD wood. All sugars were identified and quantified with HPAEC. (-)-Epicatechin concentration was determined from GC-FID results and the amount of total soluble aromatics was determined from UV-absorbance at 280 nm. Literature comparisons include various extraction mediums and methods.

Compound	Content (mg g^{-1} dry wood)				
	DE1	DE2	FE1	FE2	Literature
Total extractives	**34.10**	**31.94**	**39.64**	**33.04**	**8 - 50** [61] *
Total soluble sugars	**17.52**	**14.58**	**15.84**	**14.23**	**15** [2] **
Sucrose	-	6.15	9.39	8.68	12 [2] **
Monosaccharides	**17.52**	**8.43**	**6.45**	**5.56**	**3** [2] **
Galactose	0.41	0.07	0.09	0.06	0.34 - 0.72 [16]
Glucose	6.79	3.88	2.98	2.57	1.4 [2] **
Xylose	0.04	-	-	-	0.29 - 0.49 [16]
Fructose	10.29	4.47	3.38	2.92	1.4 [2] **
Soluble aromatics	**2.05**	**4.02**	**5.83**	**5.15**	**-**
(-)-Epicatechin	-	0.56	1.03	0.99	0.03 - 0.04 [16]
Unidentified	**14.54**	**13.34**	**17.97**	**13.65**	**-**

* Compiled from multiple studies by Routa et al.[61]

** Felled in early November, seasonal variation is significant

Compared to typical extractive compositions available in literature, the amount of extractives in all samples was on the high side as seen in Table 4. This is expected, as the amount of soluble sugars is high during winter.[2] The amount of sugars is overall in line with an earlier study by Piispanen and Saranpää[2] although the extraction procedure was very different. The amount on (-)-epicatechin, however, is much higher in DE2 and FE samples than in a study conducted on surface of birch board by Hiltunen

et al.[16] It is likely that (-)-epicatechin does not withstand the vacuum drying process very well compared to immediate acetone extraction.

Calculation examples are provided in Appendix I and chromatograms for HPAEC and GC in Appendix II.

7. CONCLUSION

The composition of extractives in birch wood is affected by storage time and drying before extraction, especially if the specific surface area of the wood is large. The amounts of (-)-epicatechin and sucrose diminished significantly in birch sawdust during storage when moisture was present and less when the sawdust was air dried immediately after sawing. A correlation between the changes in the chemical composition of the extractives and a change in the color of the sawdust was found. It was also found that the color change was not significantly reverted by an extraction with 80% acetone, suggesting that the compounds responsible for discoloration were either chemically bound to wood polymer matrix or otherwise insoluble in acetone.

Reactions that occurred in the sawdust during drying are possibly both enzymatic and autoxidative, and distinction between the two is not possible with this experimental setup. It is, however, clear that the immersion in 80% acetone prevented the reactions, both enzymatic and autoxidative, from proceeding and thus resulted slightly higher yield of extractives and drastically less discoloration during drying. For distinction between the enzymatic and the autoxidative reactions, a treatment to inactivate enzymes without affecting extractive compounds should be considered in further studies.

Autohydrolysis trials on sawdust samples showed that the DE batches had a larger acetone-soluble fraction after autohydrolysis than the FE batches. This would suggest the products of extractive oxidation contribute to the acetone-soluble fraction. Overall, the differences were rather small and there was a significant variation within the FE autohydrolysis batches, which makes drawing firm conclusions from the autohydrolysis data difficult. A more thorough analysis on the acetone-soluble fraction, for example with NMR spectroscopy, could be used to confirm that the condensation and polymerization products of phenolic extractives are included in that fraction in more discolored sawdust samples.

While it is clear from the discoloration and the simultaneous disappearance of phenolic extractive compounds that some chromophore compounds, most likely melanins and PA, were formed in the oxidation reactions, isolating these chromophores for qualitative and quantitative analysis would be required to confirm their presence. The role of proteins and amino acids in the discoloration reactions was also unclear and

analyzing the differences in protein contents of the extractives with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) could shed light on their involvement.

For more thorough analysis on the roles of oxygen, light, moisture and enzymes in the oxidation reactions, more controlled environments for drying the sawdust are required. Because moisture content of the sawdust seems extremely important and isolating it from other parameters is difficult this is not a trivial task. One way to regulate drying rate would be drying the sawdust in oven with controllable humidity and a possibility of drying in inert atmosphere to eliminate the effect of aerial oxygen.

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APPENDIX I

Appendix I includes calculation examples.

Determination of total soluble aromatics for FE1 from UV-spectrum:

$$c = \frac{A_{280nm,FE1} - A_{300nm,FE1}}{\varepsilon \cdot l}$$

$A_{280nm,FE1} = A_{280nm,total,FE1} - A_{280nm,solvent} = 0.752 - 0.165 = 0.587$

$A_{300nm,FE1} = A_{300nm,total,FE1} - A_{300nm,solvent} = 0.410 - 0.117 = 0.293$

$\varepsilon = 20\ dm^3\ g^{-1}\ cm^{-1}$

$l = 1\ cm$

$c = (0.587 - 0.293) / (20\ dm^3\ g^{-1}\ cm^{-1} * 1\ cm) = 0.294 / 20\ dm^3\ g^{-1} = 0.0147\ g\ dm^{-3}$

Determination of response factor and concentration of (-)-epicatechin:

Sample of (-)-epicatechin and D-glucose (Sigma Aldrich) was prepared for GC similarly to wood extractive samples. A response factor between the two was established so that the concentration of (-)-epicatechin could be correlated properly to concentration of glucose. This allowed the glucose that was present in the extractives to be used as an internal standard in GC after it was quantified with HPAEC. Response factor was calculated according to:

$RF = (c_{epi} / A_{epi}) / (c_{glu} / A_{glu})$,

where c are the concentrations of the respective compounds and A are the peak areas of the respective compounds in GC-FID chromatogram.

$c_{epi} = 273.3333$ mg l^{-1}

$A_{epi} = 428629.8$

$c_{glu} = 387.7778$ mg l^{-1}

$A_{glu} = 1077562$

$RF = (273.3333 \text{ mg } l^{-1} / 428629.8) / (387.7778 \text{ mg } l^{-1} / 1077562) = 1.772023945$

Concentration of (-)-epicatechin in extractives was then determined according to:

$c_{epi} = (A_{epi} / A_{glu}) * RF * c_{glu}$

For example, in DE2:

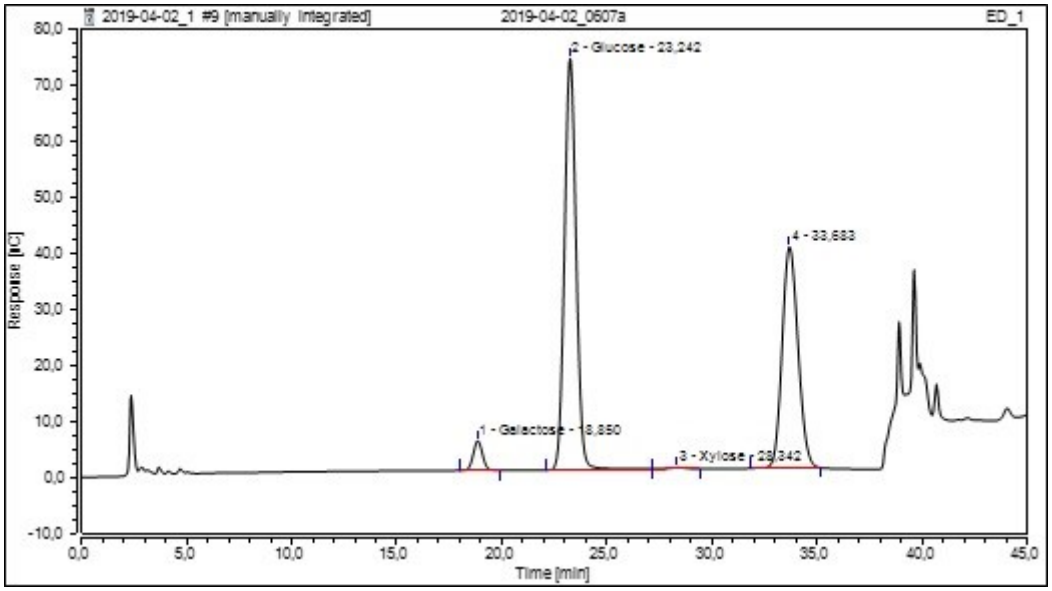
$c_{epi} = (5419.1 / 66562.8) * 1.772024 * 0.050675 \text{ g } l^{-1} = 0.007311 \text{ g } l^{-1}$

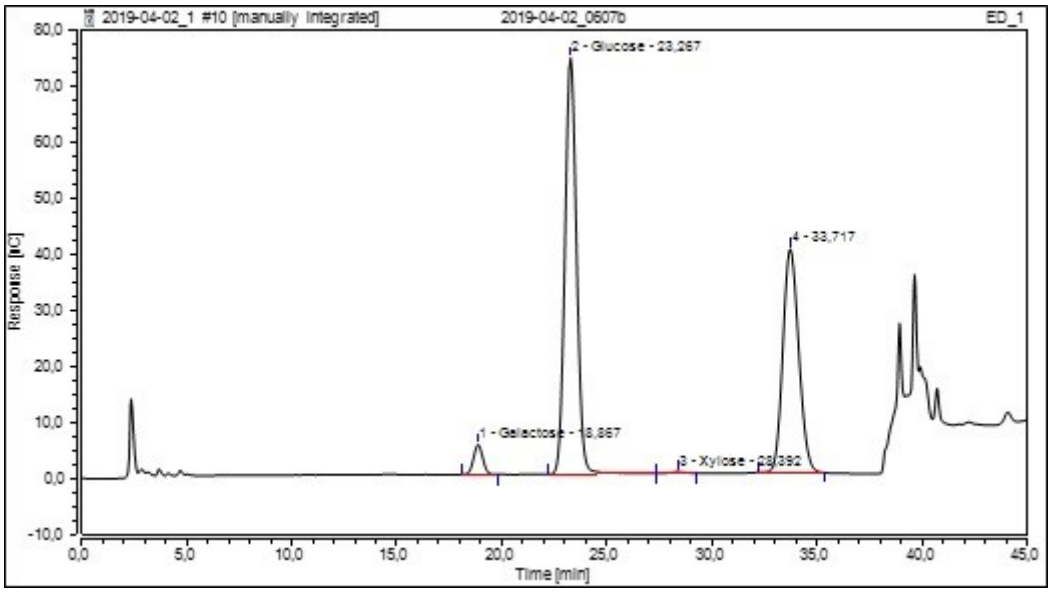
APPENDIX II

Appendix II includes chromatograms from HPAEC and GC.

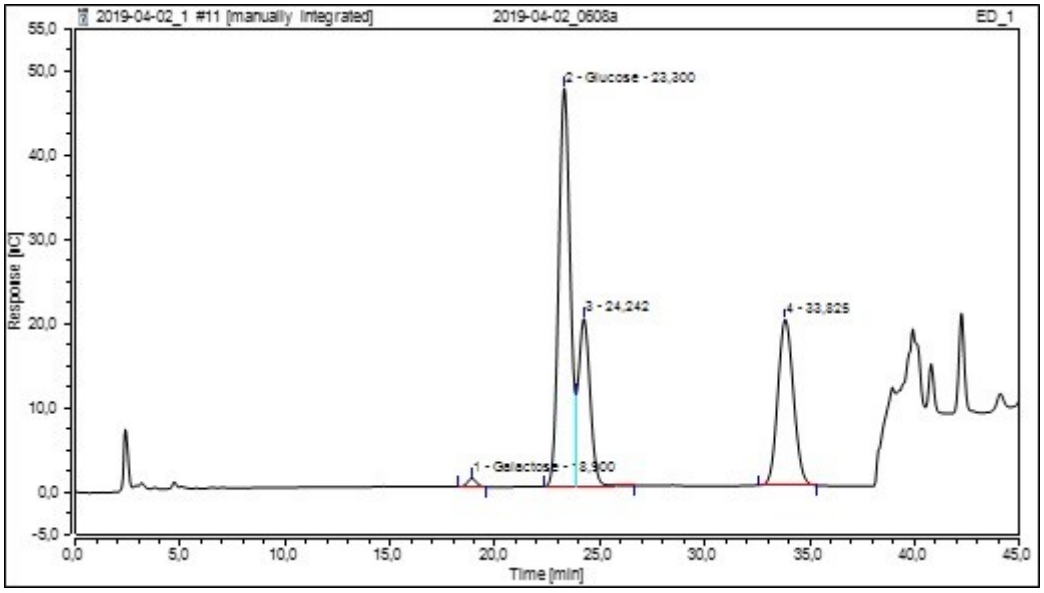
HPAEC chromatograms used for sugar quantification. All values presented in the results are an average of two runs. Sucrose and fructose were not part of the standard reference sample and identification of those compounds was done with separate calibration runs; hence, those compounds are not identified in the graphs:

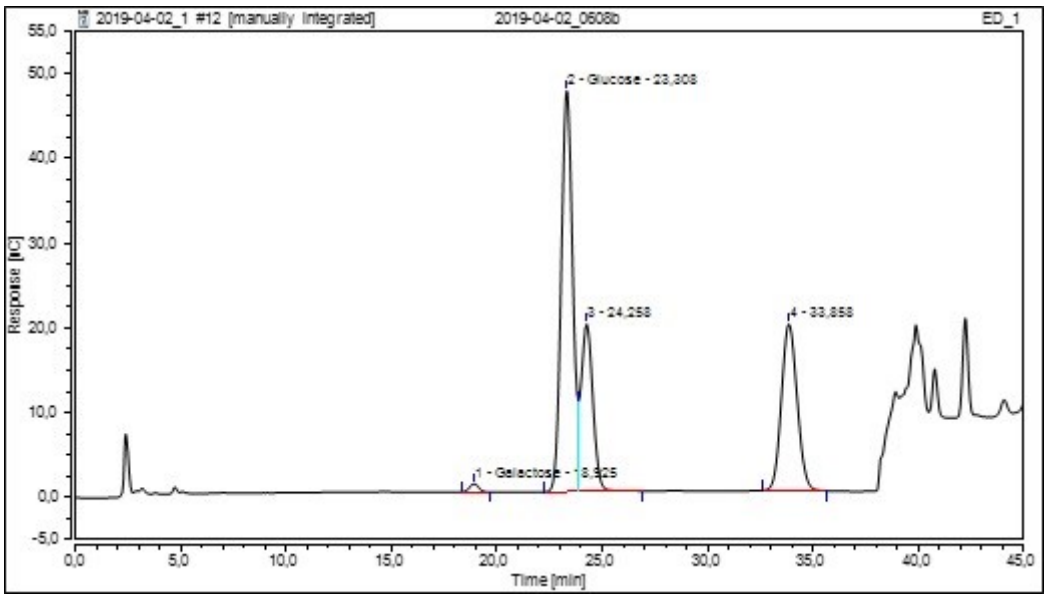
DE1, peak 4 is fructose





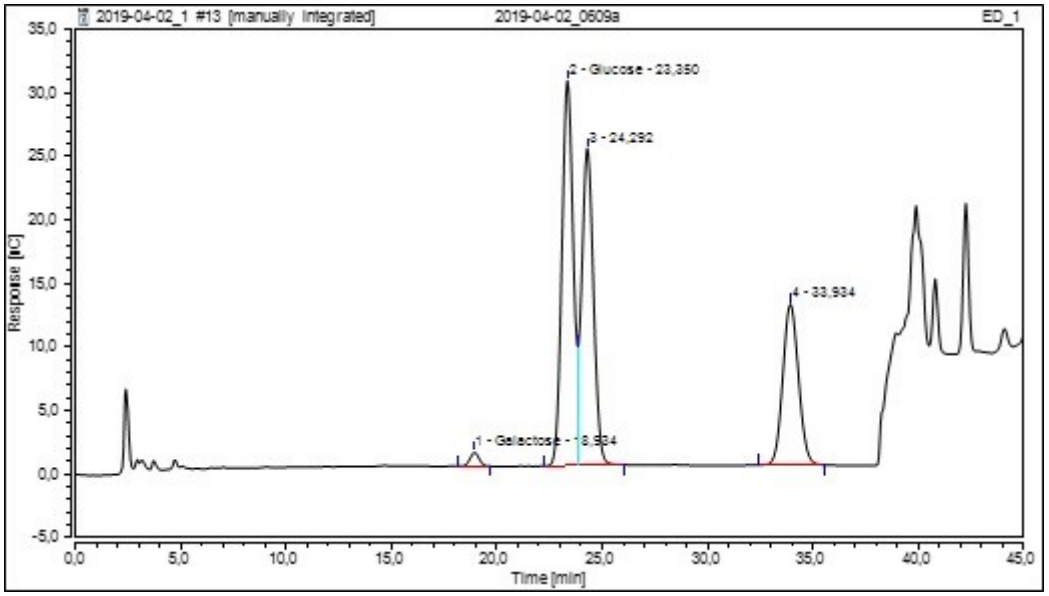
DE2, peak 3 is sucrose and peak 4 is fructose

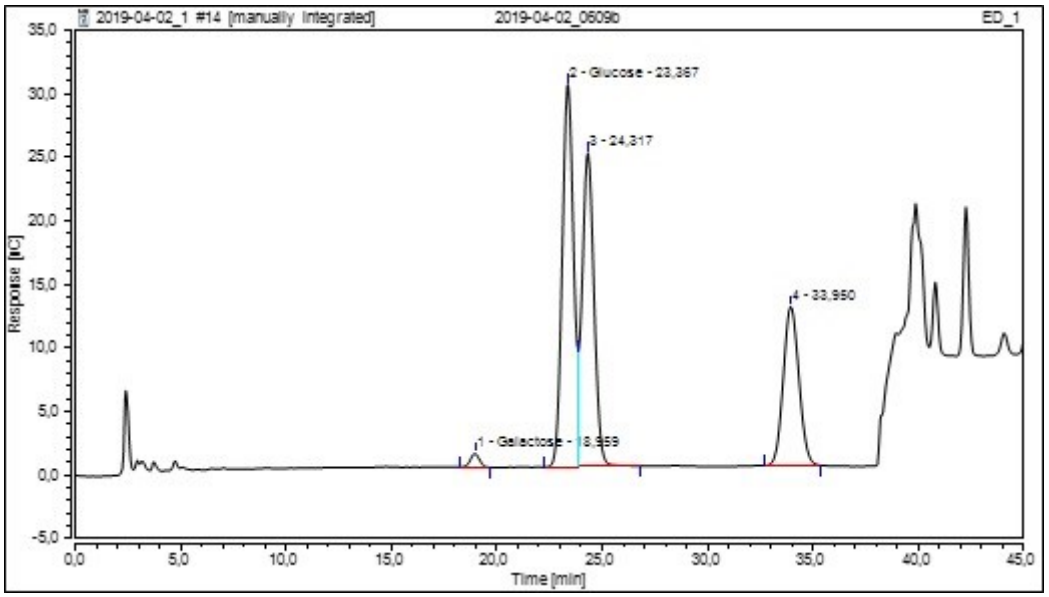




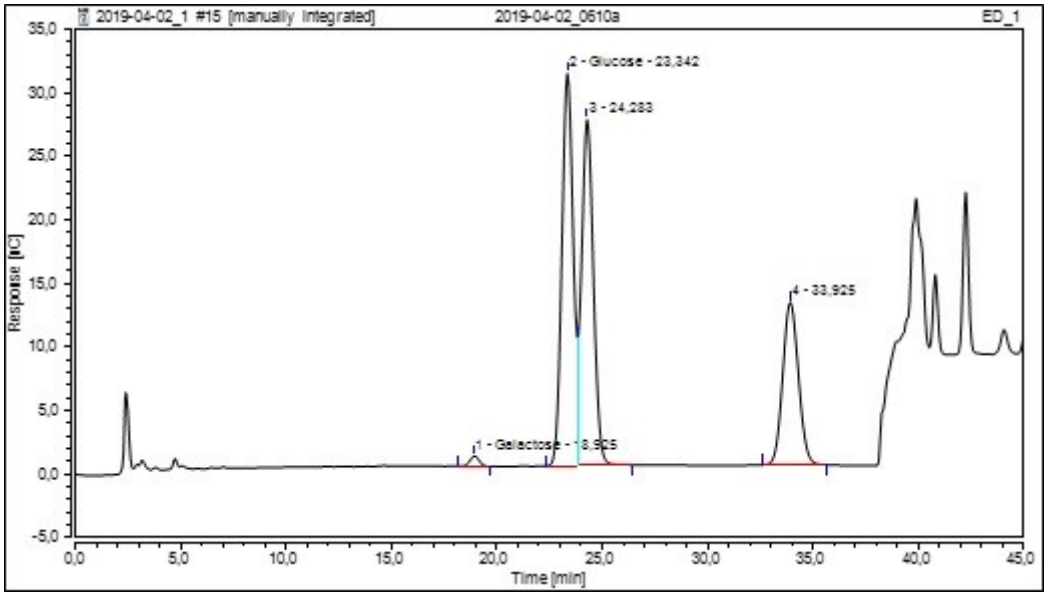
e

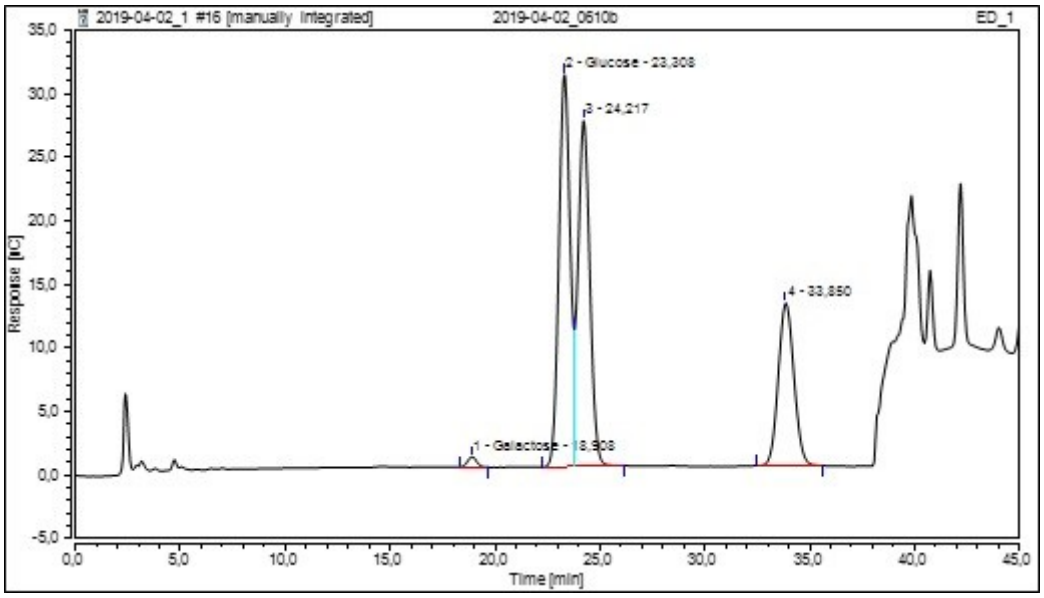
FE1, peak 3 is sucrose and peak 4 is fructose





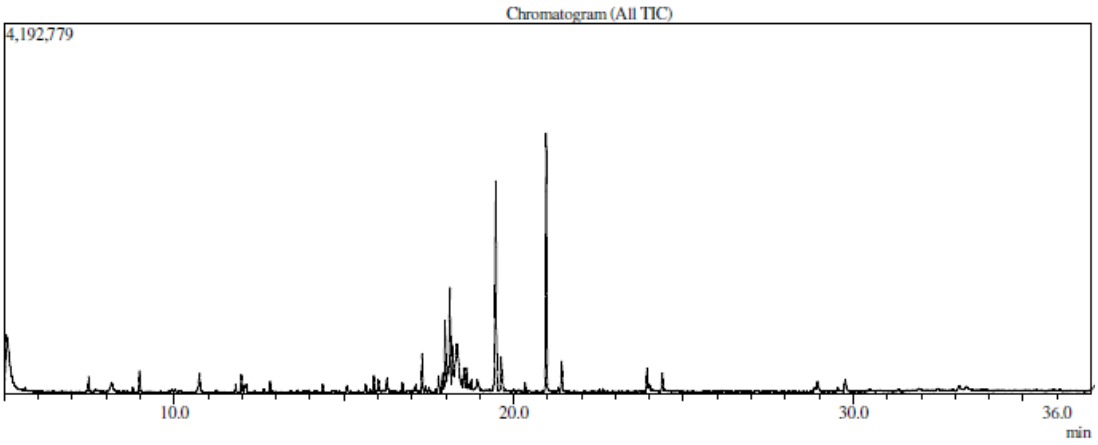
FE2, peak 3 is sucrose and peak 4 is fructose



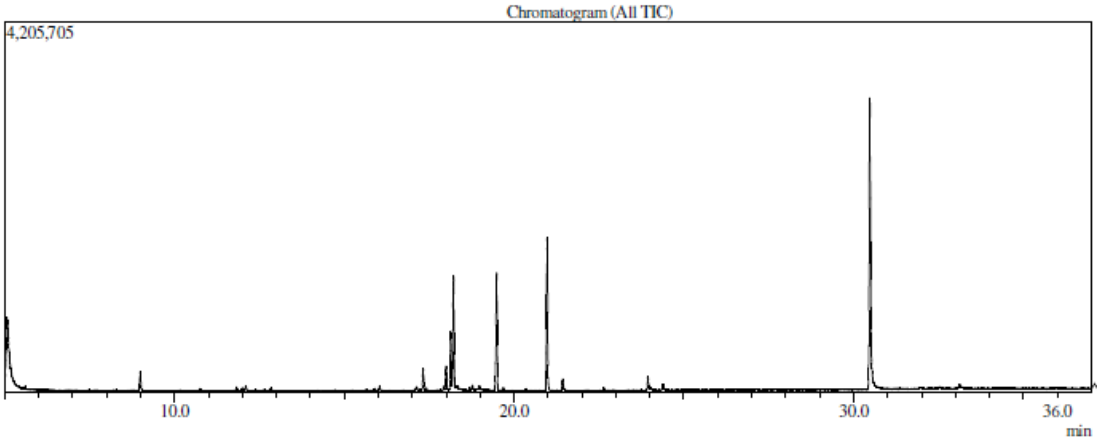


GC-MS chromatograms:

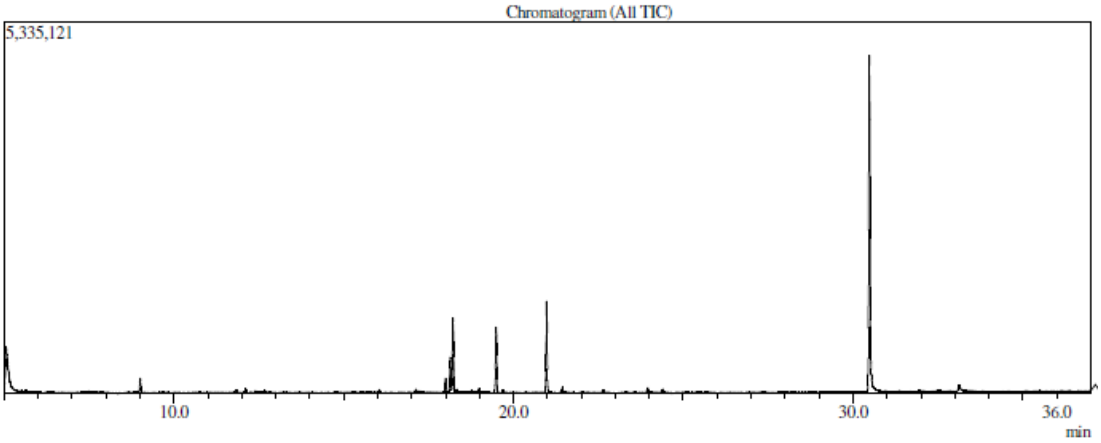
DE1



DE2



FE1


Chromatogram (All TIC)
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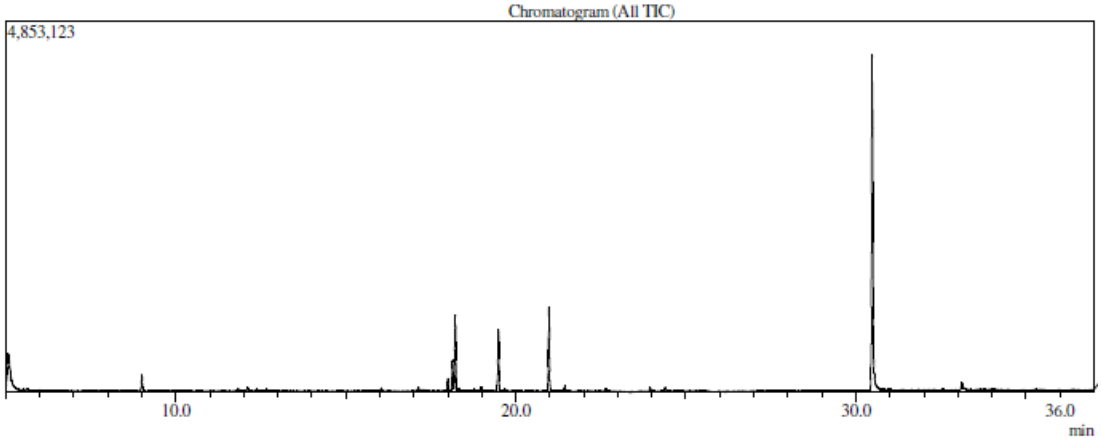
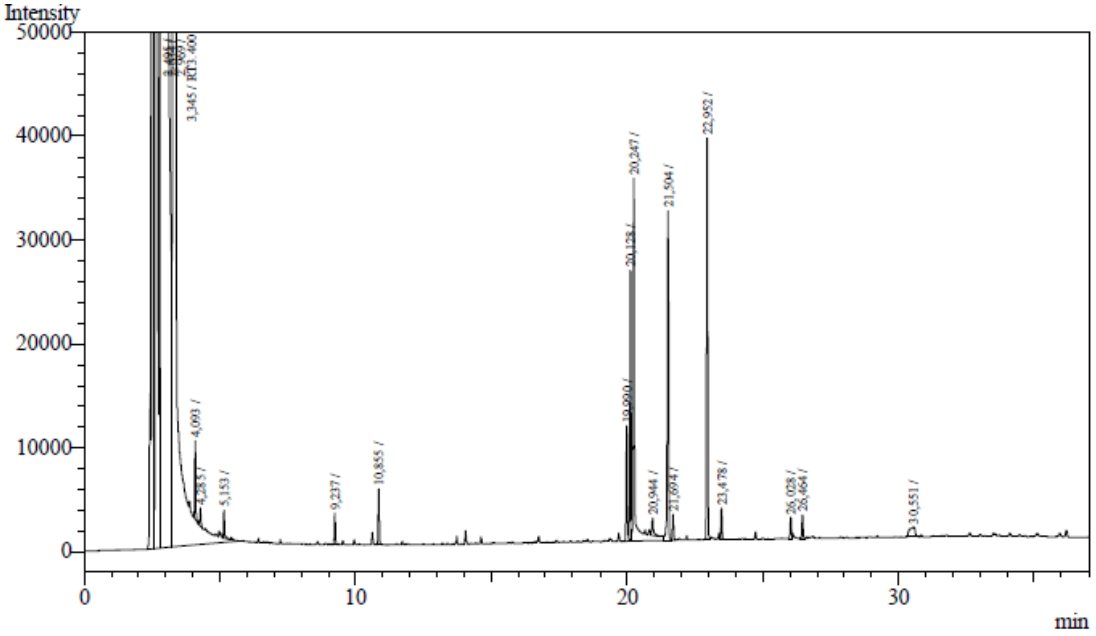
10.0
20.0
30.0
36.0
min

FE2

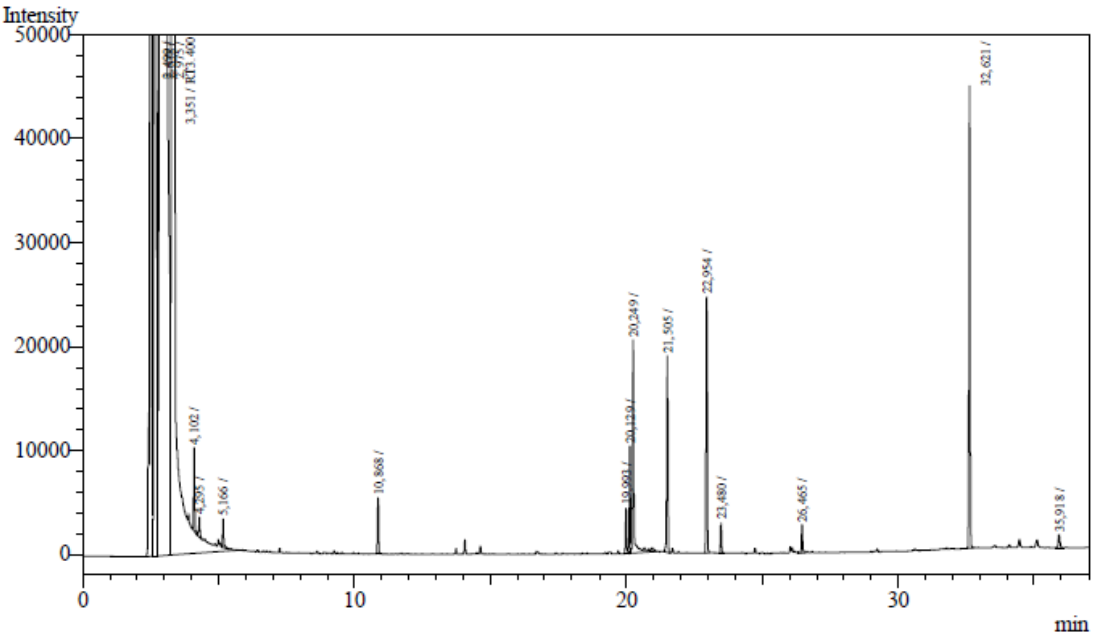

Chromatogram (All TIC)
4,853,123
10.0
20.0
30.0
36.0
min

GC-FID chromatograms:

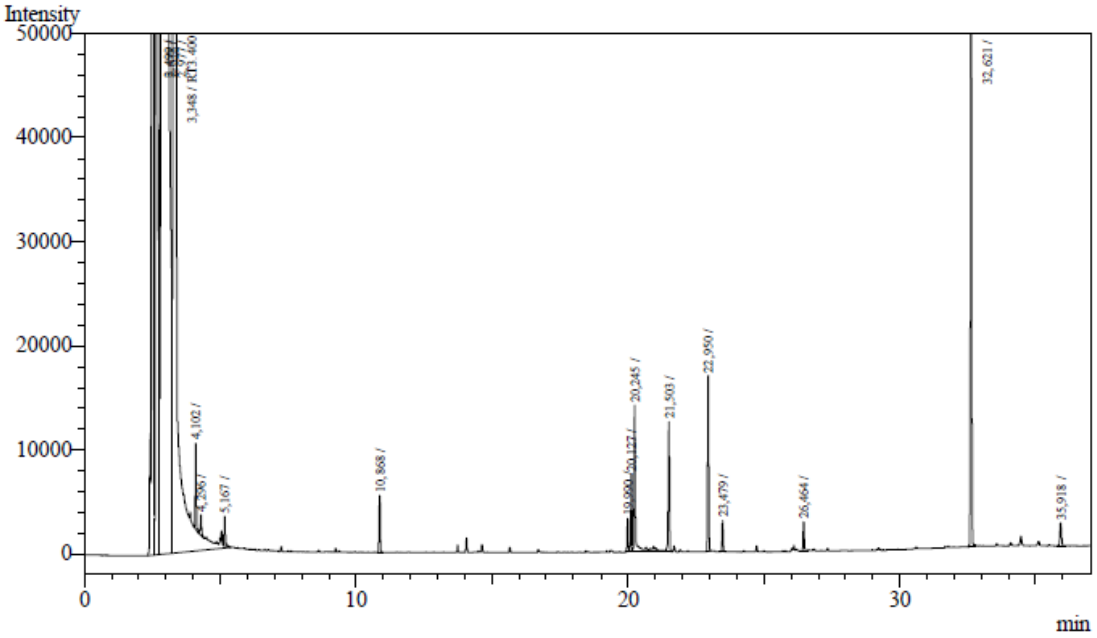
DE1



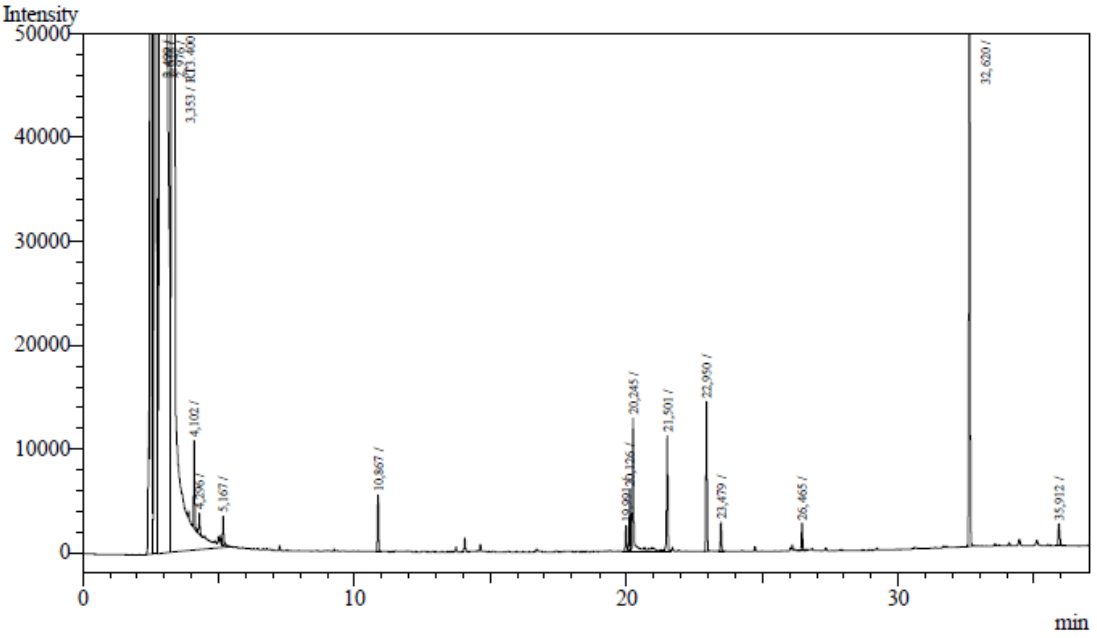
DE2



FE1


Intensity
50000
40000
30000
20000
10000
0
3,348 / RD3 466
4,102 /
4,286 /
5,167 /
10,868 /
20,245 /
21,503 /
22,950 /
23,479 /
26,464 /
32,621 /
35,918 /
0
10
20
30
min

FE2


Intensity
50000
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3,353 / RD3 466
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4,286 /
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21,501 /
22,950 /
23,479 /
26,465 /
32,620 /
35,912 /
0
10
20
30
min